Kaposi's Sarcoma–Associated Herpesvirus Infection of Bone Marrow Dendritic Cells from Multiple Myeloma Patients

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Kaposi's sarcoma–associated herpesvirus (KSHV) was found in the bone marrow dendritic cells of multiple myeloma patients but not in malignant plasma cells or bone marrow dendritic cells from normal individuals or patients with other malignancies. In addition the virus was detected in the bone marrow dendritic cells from two out of eight patients with monoclonal gammopathy of undetermined significance (MGUS), a precursor to myeloma. Viral interleukin-6, the human homolog of which is a growth factor for myeloma, was found to be transcribed in the myeloma bone marrow dendritic cells. KSHV may be required for transformation from MGUS to myeloma and perpetuate the growth of malignant plasma cells.

Multiple myeloma is the second most frequent malignancy of the blood in the United States. At any one time, 40,000 people have multiple myeloma, and ~13,000 new diagnoses are made each year (*1*). Multiple myeloma is characterized by the accumulation of malignant plasma cells in the bone marrow and the presence of a monoclonal immunoglobulin produced by the malignant plasma cells in the serum or urine or both. Normal immunoglobulin levels are profoundly suppressed. Morbidity and mortality are primarily related to skeletal, hematologic, and renal complications of the disease. Despite some advances in chemotherapeutic regimens, median survival of multiple myeloma patients has remained at about 30 months for the past several decades (*1*).

Like multiple myeloma, MGUS is characterized by a monoclonal immunoglobulin in the serum or urine and an increase of monoclonal plasma cells in the bone marrow (*1*). However, MGUS patients do not suffer from the clinical manifestations of multiple myeloma. Unlike myeloma patients, MGUS patients typically have no increased risk of infection and have normal or only modestly reduced levels of immunoglobulin other than the monoclonal protein. The prevalence of MGUS in the United States is strikingly high—about 1 million people have this disorder. One percent of people over the age of 50, 3% over age 70, and 10% over age 80 have MGUS (*1*). Because epidemiologic data repeatedly demonstrate the increasing longevity of the U.S. population, the prevalence of both MGUS and multiple myeloma are expected to rise. Importantly, 25% of patients with MGUS progress to myeloma (*2*). Despite the high prevalence of both multiple myeloma and MGUS, the molecular pathogenesis of multiple myeloma and the factors that induce transformation from MGUS to multiple myeloma remain largely unknown.

The cytokine interleukin-6 (IL-6) is a growth factor for myeloma (*3*). IL-6 may both stimulate myeloma growth and prevent apoptosis of malignant plasma cells by paracrine mechanisms (*3*). Bone marrow stromal cells provide a microenvironment for normal hematopoiesis by direct cell contact and by secretion of cytokines, including IL-6 (*4*). In myeloma, these stromal cells, which are not part of the malignant population, play a major role in mediating the paracrine stimulation of tumor cell growth (*3*, *4*).

IL-6 is also a growth factor for three other diseases: Kaposi's sarcoma (KS), pleural effusion lymphoma, and multicentric Castleman's disease. The latter two diseases are rare neoplasms of B lymphocytes. Kaposi's sarcoma–associated herpesvirus (KSHV) has been detected consistently in human immunodeficiency virus (HIV)–related and HIV-unrelated cases of all three of these diseases (*5*, *6*). KSHV was first identified in acquired immunodeficiency syndrome (AIDS)–associated KS in 1994 (*7*). KSHV is a gammaherpesvirus, related to Epstein-Barr virus and herpesvirus saimiri. A causative role for KSHV in KS is suggested by serologic data, which reveal that seroconversion to antibodies to KSHV latent nuclear antigens precedes the development of KS (*8*). KSHV has not been detected in the malignant cells of other hematologic malignancies, including myeloma, acute and chronic leukemias, Hodgkin's disease, and non-Hodgkin's lymphomas (*9*). Interestingly, a homolog to the human IL-6 has recently been identified in the KSHV genome (*10*). Of particular note, this viral IL-6 (vIL-6) retains biologic activity as demonstrated by its ability to support the growth of the murine plasmacytoma cell line B9, which undergoes apoptosis in the absence of IL-6 (*10*). On the basis of these data and the IL-6–mediated paracrine stimulation of myeloma by bone marrow stromal cells, we sought to identify KSHV in the bone marrow stromal cells of multiple myeloma and MGUS patients and to determine if this virus could play an oncogenic role in these disorders.

Using polymerase chain reaction (PCR) to amplify the $KS330_{233}$ sequence of KSHV as previously described (*7*), we evaluated 100 ng of DNA (equivalent to the amount of DNA from 15,000 cells) from bone marrow mononuclear cells and bone marrow stromal cells from multiple myeloma patients for the presence of KSHV (*11*, *12*). The bone marrow mononuclear cells contain a significant proportion of malignant plasma cells (11 to 90% in our cohort), whereas the bone marrow stromal cells are essentially devoid of malignant cells. The $KS330_{233}$ sequence was detected in 15 of 15 bone marrow stroma cell DNA samples from multiple myeloma patients (5 patients previously treated with chemotherapy for multiple myeloma and 10 untreated patients), but in 0 of 23 fresh myeloma bone marrow mononuclear cell DNA samples (Fig. 1A). The absence of KSHV in bone marrow mononuclear cells indicates that the virus does not infect the malignant clone, a finding that has been reported (*9*). We also evaluated the bone marrow stromal cells of patients with MGUS for the presence of KSHV. We detected the $KS330_{233}$ sequence in the bone marrow stromal cells of two of eight MGUS patients, but not in the bone marrow mononuclear cells of any of these patients.

To confirm the specificity of our findings, we evaluated the bone marrow stromal cell DNA from 10 normal individuals and from 16 patients with other malignancies

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[14 lymphoma (2 Hodgkin's disease and 12 non-Hodgkin's lymphoma) patient, 1 acute myelogenous leukemia patient, and 1 adenocarcinoma patient with bone involvement]. The $KS330_{233}$ amplification product was not detected in the bone marrow stromal cell DNA from any of these 26 samples. Likewise, the bone marrow mononuclear cells from these patients contained no detectable virus. Five of the 12 non-Hodgkin's lymphoma patients had gross bone marrow involvement with tumor.

Southern (DNA) blotting of the PCR amplification products with a ^{32}P end-labeled probe internal to the $KS330_{233}$ sequence (*13*) corroborated our results (Fig. 1B). All PCR-positive lanes hybridized to this probe, whereas none of the PCR-negative lanes yielded a positive hybridization signal. Moreover, DNA sequencing of the $KS330_{233}$ PCR amplification products from two myeloma bone marrow stromal cell samples yielded one– to four–base pair (bp) differences from the sequence originally reported (*14*). If the PCR products were a result of PCR contamination, the sequences we obtained from different patients would have been identical. In addition, PCR amplification was also performed independently with vIL-6–specific primers, which yielded the same results as those obtained with the $KS330_{233}$ primers (*15*).

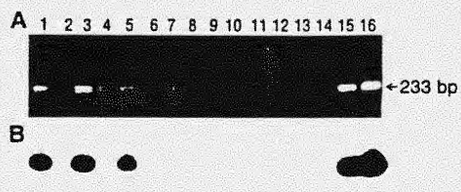

Fig. 1. Representative results of DNA PCR amplification of the $KS330_{233}$ sequence. (**A**) Agarose gel of the 233-bp PCR amplification products. The 233-bp PCR product (arrow) is detected in the lanes representing myeloma bone marrow stromal cells (lanes 1, 3, 5, and 15), but not in the lanes of myeloma bone marrow mononuclear cells (lanes 2, 4, and 6; same patients as lanes 1, 3, and 5, respectively) or bone marrow stromal cells of a non-Hodgkin's lymphoma patient (lane 7) and normal individuals (lanes 8 to 13). The positive control (lane 16) was the PCR product from the pleural effusion lymphoma cell line KS-1 (*6*). Placental DNA was the negative control (lane 14). To ensure reproducibility, we repeated PCR up to three times. Identical results were obtained each time. (**B**) The agarose gel in (A) was transferred to a nylon membrane and hybridized to a ^{32}P end-labeled 25-bp oligonucleotide probe, the sequence of which is internal to that of the 233-bp PCR product.

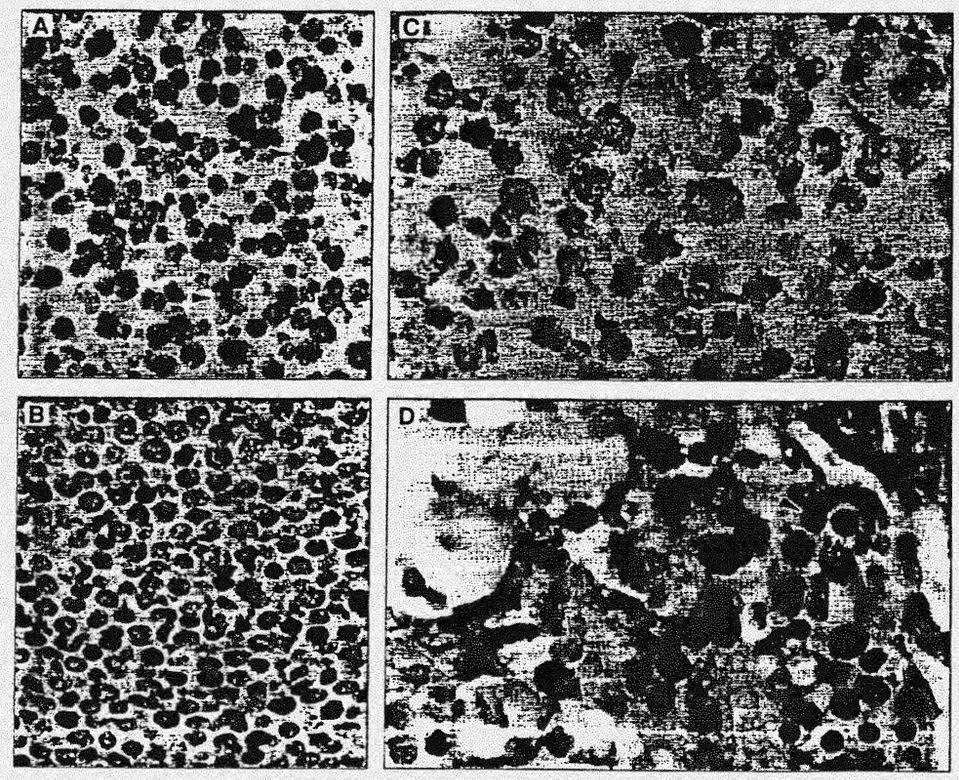

Fig. 2. In situ hybridization with a biotinylated KSHV probe. (**A**) In situ hybridization with the biotinylated KSHV probe of the pleural effusion lymphoma cell line KS-1 shows nuclear and cytoplasmic staining (brown). Chromosomes in mitotic cells are stained (arrowheads). (**B**) The acute myelogenous leukemia cell line HL60 is negative for KSHV. (**C**) The bone marrow stromal cells from a multiple myeloma patient are positive for KSHV in a nuclear and cytoplasmic pattern (brown). (**D**) In situ hybridization of a bone marrow aspirate from the same multiple myeloma patient as in (C) demonstrates malignant plasma cells (arrowheads), which are negative for KSHV. Original magnifications ×400.

In situ hybridization (*16*) was performed as another confirmatory technique to document the specificity of the KSHV infection of myeloma bone marrow stroma. In situ hybridization with a probe specific for KSHV sequences demonstrated nuclear and cytoplasmic staining of myeloma bone marrow stromal cells (Fig. 2) In situ hybridization on myeloma bone marrow stromal cells with a probe specific for cytomegalovirus and with an irrelevant probe (specific for plasmid DNA) were negative, as was in situ hybridization with the KSHV probe on normal stroma, malignant plasma cells, and the HL60 cell line (Fig. 2). To determine if signals were from specific hybridization to DNA and RNA, we pretreated slides with deoxyribonuclease (DNase) and ribonuclease (RNase), which abolished the staining of myeloma bone marrow stromal cells by the KSHV probe.

To eliminate any bias in the PCR results, we took several precautions. PCR was performed three times in a blinded fashion; the technician was unaware of the patient identities or diagnoses. All bone marrow stromal cells were processed in the same hood and cultured in the same incubator simultaneously. A master mix was used to perform the blinded PCR, so that KSHV contamination of the PCR reagents cannot be invoked as an explanation for our findings. Inadequate PCR or degraded DNA was not the reason for lack of PCR detection of KSHV, because all negative samples yielded appropriately sized amplified product with β-actin primers (*15*).

Although bone marrow stromal cells are derived from the original bone marrow mononuclear cell population (*11*), they make up a small fraction of the total bone marrow cellular compartment. Moreover, bone marrow aspirates are heavily contaminated with peripheral blood, a phenomenon that greatly dilutes the frequency of bone marrow stromal cells in fresh samples. As stromal cells are adherent cells, they are not readily removed during bone marrow aspiration or expelled from the instruments used to obtain bone marrow. Consequently, although the PCR amplification detected KSHV in DNA samples obtained from a population enriched for bone marrow stromal cells, the assay did not have the sensitivity to detect KSHV in fresh bone marrow mononuclear cells.

In general, bone marrow stromal cells are thought to include fibroblasts, macrophages, and endothelial cells. By immuno-

histochemistry (*17*), bone marrow stromal cells were negative for CD31, an endothelial cell marker (Fig. 3A), and CD34, a nonspecific but sensitive marker for fibroblasts. The bone marrow stromal cells were positive for CD68, a macrophage marker; fascin, a 55-kD actin-bundling protein highly restricted to dendritic cells (a type of macrophage); and CD83 (*18*), another dendritic cell marker (Fig. 3, B to D). These cells were positive for vimentin and negative for lysozyme and CD1a, a Langerhans cell marker. Of note, the morphology of the cells depicted in Fig. 3 has been perturbed by the trypsinization process, which causes the adherent dendritic cells to take on a more spherical shape. Ultrastructural evaluation (*19*) demonstrated the stromal cells to contain abundant cytoplasm with numerous primary and secondary lysozymes and short cytoplasmic processes, all characteristics of macrophages (Fig. 4). The immunohistochemical and ultrastructural analyses yielded identical results for the bone marrow stromal cells obtained from multiple myeloma and MGUS patients as those for patients with other malignancies and normal individuals. On the basis of our findings, KSHV appears to infect a subset of macrophages, namely, dendritic cells.

To establish a link between the presence of KSHV in myeloma bone marrow stromal cells and the paracrine stimulation of tumor growth by cytokines produced by these stromal cells, we attempted to detect vIL-6 RNA transcripts in myeloma bone marrow stromal cells. Using vIL-6–specific primers to perform reverse transcriptase–PCR (RT-PCR) (*20*), we demonstrated the presence of amplified vIL-6 product in three of three myeloma bone marrow stromal cell samples, but in zero of two KSHV-negative stromal samples obtained from normal individuals (Fig. 5). Therefore, vIL-6 may contribute to the mechanism whereby bone marrow stromal cells infected with KSHV promote myeloma growth.

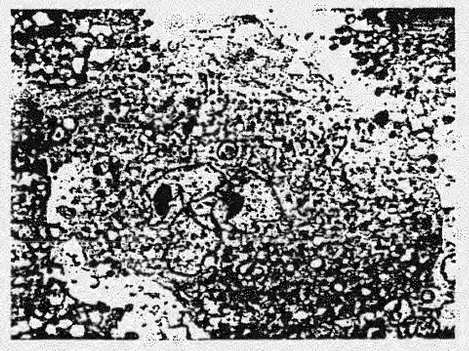

Fig. 4. Ultrastructural appearance of a stromal cell cultured from multiple myeloma bone marrow. The cell contains abundant cytoplasm with numerous primary and secondary lysozymes and short cytoplasmic processes, all characteristics of macrophages. (Uranyl acetate, lead citrate, original magnification ×2850.)

Here we have shown that KSHV infected the dendritic cells in the bone marrow of all the multiple myeloma patients studied. Malignant plasma cells were not infected with this virus. The virus also infected the dendritic cells of one-fourth of the MGUS patients studied. The detection of KSHV in the bone marrow stromal cells of a significant proportion of MGUS patients has potential implications for the risk of transformation from MGUS to multiple myeloma. Specifically, those MGUS patients whose

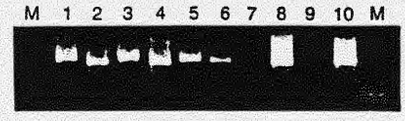

Fig. 5. RT-PCR of vIL-6 performed on bone marrow stromal cell total RNA from three multiple myeloma patients and two normal individuals. Appropriately sized 695-bp vIL-6 products were obtained from the myeloma bone marrow stromal cells (lanes 1, 3, and 5) but not the normal bone marrow stromal cells (lanes 7 and 9). RT-PCR with β-actin primers on bone marrow stromal cell total RNA from the same individuals (lanes 2, 4, 6, 8, and 10) yielded appropriately sized 650-bp products. M, 123-bp molecular size ladder.

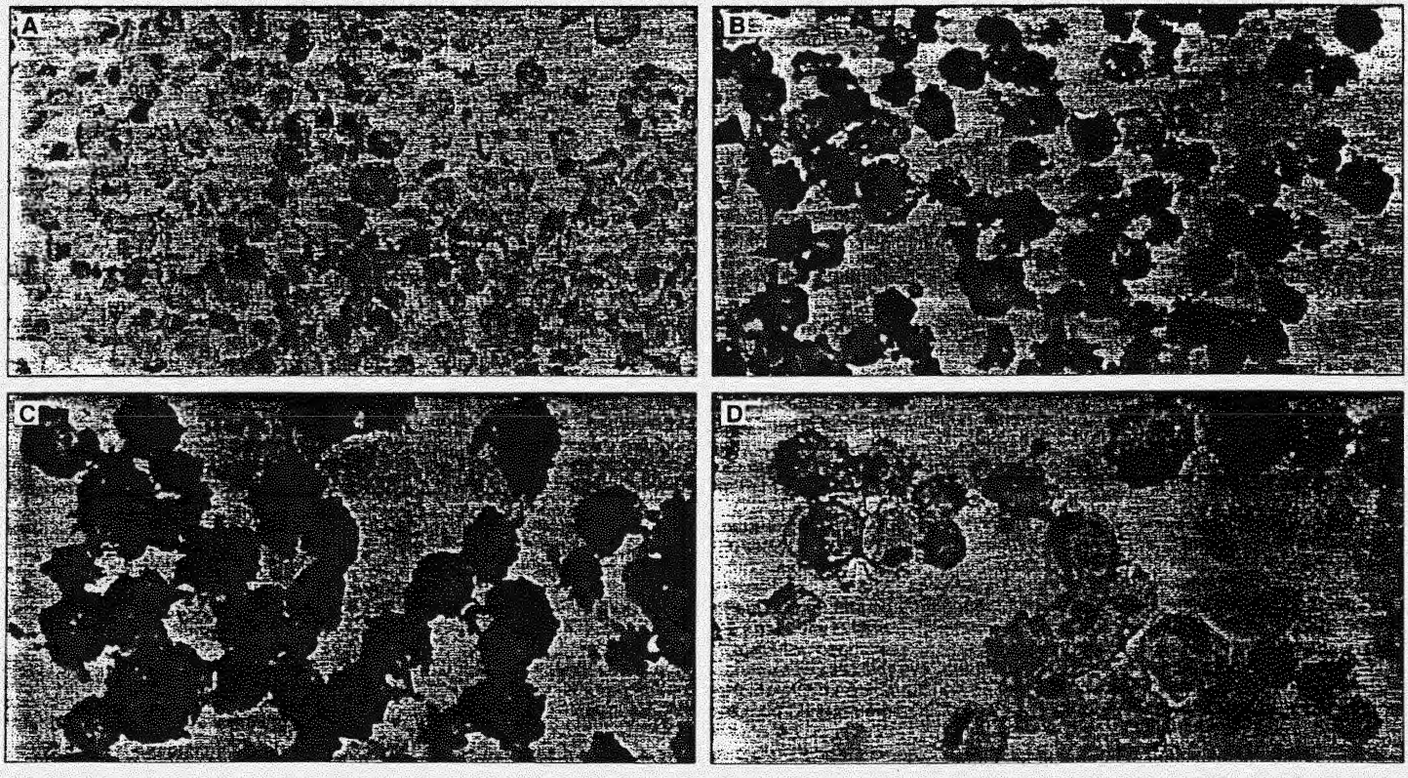

Fig. 3. Immunohistochemistry of multiple myeloma bone marrow stromal cells. (**A**) Stain for CD31, an endothelial cell marker is negative. (**B**) Stain for CD68, a specific macrophage marker, is strongly positive (brown). (**C**) Fascin stain, a dendritic cell marker, is positive (red). (**D**) Stain for CD83, another dendritic cell marker, is positive (brown). (Original magnifications ×400; counterstain with hematoxylin.)

bone marrow stromal cells are infected with KSHV may well be the subset (25%) who go on to develop multiple myeloma. Approximately 30 to 40% of MGUS patients have modestly reduced levels of one or more immunoglobulins (*21*). Thus, an immunodeficient state may account for an increased susceptibility of MGUS patients to KSHV infection. However, because the immunoglobulin amounts were normal in the two MGUS patients in whose bone marrow stromal cells we detected the virus, the detection of KSHV in myeloma bone marrow stromal cells cannot be entirely explained by the unique immunologic state due to the generalized suppression of immunoglobulin production, which is typical of multiple myeloma.

The bone marrow dendritic cells from patients with other malignancies and from normal individuals were not infected with the virus, and vIL-6, the human homolog of which is a major growth factor for malignant plasma cells, was transcribed in virally infected dendritic cells from myeloma patients. Recently, dendritic cells have been shown to play a critical role in the growth and differentiation of mature B cells and to increase (by 30- to 300-fold) the secretion of immunoglobulins G and A by B cells (*22*). Taken together, these data suggest that KSHV may play a causative role in the transformation of MGUS to multiple myeloma and the propagation of fully malignant plasma cells once myeloma has become manifest. KSHV infection of dendritic cells localizes the virus to the bone marrow microenvironment where viral genes (such as the gene encoding vIL-6) are expressed and may support myeloma growth. This study demonstrates some evidence that a virus can potentially support the growth of a malignancy by infecting a nonmalignant cell without infecting the malignant clone.

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11. Bone marrow aspirates were obtained from 23 patients with active multiple myeloma, 10 normal donors for allogeneic bone marrow transplantation, 12 non-Hodgkin's lymphoma patients, 1 acute myelogenous leukemia patient, 1 metastatic adenocarcinoma patient, and 2 Hodgkin's disease patients. All specimens were obtained after informed consent and in compliance with the Human Subjects Protection Committee. Cytospins of fresh bone marrow mononuclear cells from the 23 multiple myeloma patients, which were separated by Ficoll-Hypaque density sedimentation, demonstrated 11 to 90% malignant plasma cells. Bone marrow mononuclear cells separated by Ficoll-Hypaque density sedimentation were used to establish long-term bone marrow stromal cell cultures as described [S. M. Gartner and H. S. Kaplan, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4756 (1980)]. Bone marrow mononuclear cells were suspended in 10 ml of Iscove's modified Dulbecco's medium (Irvine Scientific, Santa Ana, CA), supplemented with 10% fetal calf serum (Gemini Bio-Products, Calabasas, CA), 10% horse serum (Gemini Bio-Products), 200 mM L-glutamine (Gemini Bio-Products), penicillin (100 U/ml, Irvine Scientific), and streptomycin (100 μg/ml, Irvine Scientific) in 75-cm^2 flasks (CoStar, Cambridge, MA). Cells were incubated at 37°C in a CO_2 incubator. The growth medium was changed every week. Once a confluent, adherent cell monolayer developed, the monolayer was washed three times with phosphate-buffered saline (PBS). The adherent bone marrow stromal cells were harvested with 0.5% trypsin in Hanks' buffered saline solution (Irvine Scientific), washed with PBS, and collected by centrifugation.
12. PCR was performed with 100 ng (~15,000 cells worth) of genomic DNA from bone marrow stromal cells (see below) or bone marrow mononuclear cells, the component of bone marrow that contains the malignant plasma cells. A total of 45 cycles of PCR amplification was performed on all samples to detect the $KS330_{233}$ sequence as described (*7*). The positive control was genomic DNA from the pleural effusion lymphoma cell line KS-1 (*6*), and the negative control was placental DNA (Stratagene, La Jolla, CA). PCR product (10 μl) was electrophoresed on a 1% agarose gel impregnated with ethidium bromide and then photographed. PCR with vIL-6 primers was performed in a similar fashion (*10*). PCR with β-actin primers (forward primer, 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′; reverse primer, 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′) was performed on all samples that were PCR-negative with the $KS330_{233}$ primers. The PCR amplification was repeated up to three times to assure the reproducibility of the results. Samples were also evaluated in a blinded fashion (the source of bone marrow stromal cells was unknown by the technician).
13. Southern (DNA) blot transfer was performed from the gel obtained from the PCR products. A ^{32}P end-labeled probe internal to the $KS330_{233}$ sequence was used for hybridization as described (*7*).
14. The $KS330_{233}$ PCR product was cloned into the pCR 2.1 vector (Invitrogen, San Diego, CA) and sequenced with the Sequenase Version 2.0 Sequencing Kit (Amersham Life Science, Cleveland, OH) according to the manufacturers' instructions.
15. M. B. Rettig *et al.*, data not shown.
16. Serial paraffin sections were mounted on glass slides, deparaffinized, and immersed in 3% hydrogen peroxide to quench endogenous peroxidase activity. After proteinase digestion for 30 min at 37°C, slides were incubated sequentially with 10% paraformaldehyde solution for 5 min at room temperature and with glycine solution at 37°C for 5 min. The slides were then dehydrated and air-dried, and a cover slip was applied and then heated at 95°C for 4 min to denature viral DNA. Sections were incubated for 18 hours at 37°C with a biotinylated probe diluted 1:100 to 1:300 in 50% formamide, 2× standard saline citrate (SSC), Denhardt's solution, dextrose sulfate, and salmon sperm DNA. The probe was in sense orientation with the following sequence: 5′-TGCAGCAGCTGTTGGTGTACCACATCTACT-3′; T denotes biotinylated thymidine. The sequence of this probe was identical to that of the probe used for Southern blotting, except for the addition of 5 bp to the 3′ end (*7*). Slides were then washed in tris-buffered saline and 0.02% SDS and incubated with a monoclonal mouse antibody to biotin (Dako, Carpinteria, CA) followed by horseradish peroxidase–conjugated rabbit antibody to mouse and goat antibody to rabbit immunoglobulins (Dako). The reaction product was visualized by means of the diaminobenzidene reaction. To determine if in situ hybridization signals were from specific hybridization to DNA and RNA, we pretreated slides with RNase (1 mg/ml) (DNase-free, Sigma, St. Louis, MO) and DNase (1 mg/ml) (RNase-free, Boehringer Mannheim, Indianapolis, IN).
17. After trypsinization, bone marrow stromal cells were pelleted and fixed with formalin. Pellets were embedded in paraffin and then sectioned. Staining for CD31, CD34, CD68, CD1a, CD83, vimentin, and lysozyme was performed on deparaffinized sections with monoclonal antibodies (all antibodies were from Dako, Carpinteria, CA, except antibody to CD83, which was from Immunotech, Marseille, France) by using immunoperoxidase methods as described [J. Ho *et al.*, *Appl. Immunohistochem.* **2**, 282 (1994)]. Staining for fascin was performed as described [G. S. Pinkus *et al.*, *Am. J. Pathol.* **150**, 543 (1997)].
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19. Cells were fixed in glutaraldehyde, pelleted, and embedded for electron microscopy as described [J. W. Said *et al.*, *Blood* **87**, 4937 (1996)]. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a transmission electron microscope (JEOL, Peabody, MA).
20. Total RNA was prepared with RNAzol (TelTest, Friendswood, TX) according to the manufacturer's instructions. First-strand synthesis was performed at 42°C for 1 hour with 5 μg of total RNA. PCR was done in 200 μM for each deoxynucleotide triphosphate and 5 pmol of each vIL-6 primer (*7*). The PCR sequence was as follows: 44 cycles at 58°C for 1 min, 72°C for 1.5 min, 94°C for 1 min with a 5-min initial denaturation at 95°C, and a final 5-min elongation step. PCR product (10 μl) was electrophoresed on a 1% agarose gel impregnated with ethidium bromide and then photographed. The expected PCR product is 695 bp. RT-PCR with β-actin primers (*12*) was performed on samples (3 μg of total RNA) that were RT-PCR negative with the vIL-6 primers.
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